Genetically-encoded imaging tools for investigating cell dynamics at a glance

Méghane Sittewelle, Nuria Ferrandiz, Mary Fesenko and Stephen J. Royle*

Centre for Mechanochemical Cell Biology, Division of Biomedical Sciences, Warwick Medical School, University of Warwick, Coventry, CV4 7AL, UK

s.j.royle@warwick.ac.uk

ORCiD:
MS 0000-0002-9383-6653
NF 0000-0002-7410-3470
MF 0000-0002-0407-6374
SJR 0000-0001-8927-6967

key words:
imaging, microscopy, optogenetics, chemically-induced heterodimerization

The biology of a cell is the sum of many highly dynamic processes, each orchestrated by a plethora of proteins and other molecules. Microscopy is an invaluable approach to spatially and temporally dissect the molecular details of these processes. Hundreds of genetically encoded imaging tools have been developed that allow cell scientists to determine the function of a protein-of-interest in the context of these dynamic processes. Broadly, these tools fall into three strategies: observation, inhibition and activation. Using examples for each strategy, in this 'Cell science at a glance' and the accompanying poster, we provide a guide to using these tools to dissect protein function in a given cellular process. Our focus here is on tools that allow rapid modification of proteins of interest and how observing the resulting changes in cell states are key to unlocking dynamic cell processes. The aim is to inspire the reader's next set of imaging experiments.

Introduction

Cell biology involves understanding cellular processes at the molecular level. For a given pathway or process, we would like to know what molecules are involved and how they work together to generate a particular function. All cellular processes are highly dynamic, and microscopy is a powerful approach to investigate the molecular players in a particular pathway, reporting the spatial and temporal details that are not revealed by biochemical approaches. Cell scientists now have many wonderful genetically encoded imaging tools to deploy and unpick the functions of the molecules in a process, with many more methods being generated all the time. Here we focus on tools that allow us to dynamically probe protein function in cellular processes that we classified into three different strategies: observation - tools that allow us to observe protein function in as close to native state as possible; inhibition - tools that allow us to inhibit protein function and observe the effect on cellular processes; and activation - tools that allow us to activate proteins to confer function or reconstitute it inside the cells.

With all three strategies, the approach used needs to be tuned to the timing of the cellular process under investigation. Any switches in cellular state must occur at least as rapidly as the process being studied (Box 1). Common approaches used are chemogenetic (using a chemical compound together with a compatible genetically encoded protein domain) or optogenetic (using light to trigger a change in a protein domain), often to induce heterodimerization (Box 2). Generally, protein domains can be fused to one another in order to build a new protein with the desired characteristics to investigate your protein of interest (POI). Here, and in the poster, we provide a non-comprehensive overview of these tools, highlighting exemplar uses for each strategy and covering design principles to consider when designing your own experiments.

Observation

To understand the dynamics of proteins in a cellular process, a number of approaches allow us to image cells in an analogous way to biochemical “pulse chase” experiments, where a labelled compound (pulse) is chased with unlabeled compound to understand dynamics. Fluorescent protein tags that allow photoactivation, photoswitching, photobleaching or photoconversion mean that we can label subsets of tagged protein and study their behavior. Two popular methods to do this are fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP). Here, a POI is tagged with a fluorescent protein and bleached using concentrated illumination in a subregion of the cell (see poster – “Protein dynamics”). The dynamics of recovery, or the loss of cellular signal, can be analyzed to understand the mobility of the POI (Chudakov et al., 2010). An alternative is to selectively label the cell surface population of receptors and monitor their trafficking following endocytosis. This is possible with extracellular HaloTags and the use of cell-impermeable dyes (Huet-Calderwood et al., 2017). HaloTag is a 33 kDa tag derived from a bacterial enzyme that can be covalently labelled with a fluorescent dye. Labeling can also be achieved using a chemical heterodimerization approach (Box 2) to “FerriTag” POIs so that they can be observed by both light and electron microscopy (Clarke and Royle, 2018). Here, an engineered ferritin particle (visible by EM), with multiple copies of FRB and the red fluorescent protein mCherry (visible by fluorescence microscopy), can be inducibly attached to an FKBP-tagged POI in live cells.

Trafficking of proteins can also be monitored by Retention Using Selective Hooks (RUSH), a system where a POI can be released synchronously from a holding compartment in the cell (Boncompain et al., 2012). With RUSH (see poster – “Protein dynamics”), a secretory

pathway POI is tagged with a streptavidin-binding peptide (SBP) and is co-expressed with an ER-resident protein that is fused to streptavidin. This ER-resident protein may have, for example, a KDEL ER-retention signal, and because SBP binds tightly to streptavidin, the POI is trapped in the ER. The SBP-streptavidin interaction can be broken by adding excess biotin. Biotin competitively displaces the SBP as it binds streptavidin with even higher affinity than SBP. The SBP-tagged POI is then released, allowing us to observe its secretion and quantify its trafficking dynamics. Biotin freely diffuses across all cell compartments and can be easily added to the media while the cells are on the microscope stage. RUSH has been useful for examining the effect of physical forces on secretion (Phuyal et al., 2022) and for observing anterograde traffic of procollagen (McCaughey et al., 2019).

Reporters

A number of fluorescent protein-based sensors can respond dynamically to changes in cellular state. One example is pHluorins, pH-sensitive mutants of GFP whose fluorescence is quenched in acidic environments (such as lysosomes) (see poster - “Reporters”). pHluorins have been used as reporters of exocytosis and endocytosis (Miesenböck et al., 1998; Merrifield et al., 2005). Another example of this is the fluorescent, ubiquitination-based cell cycle indicator (FUCCI) that switches from a green fluorescence in G1 phase to a red signal in S/G2/M-phase of the cell cycle via the tagging of two licensing factors Cdt1 and geminin that are differentially degraded (Sakaue-Sawano et al., 2008) (see poster inside the nucleus). Genetically encoded calcium indicators, such as the GCaMP family, fluoresce when calcium binds to the fused calmodulin (Chen et al., 2013). These are widely used as reporters of neuronal activity. These reporters, like others in this article, are readily transplantable and can be targeted to different cellular compartments using tried-and-tested targeting motifs (see poster – “Reporters”).
Fluorescence resonance energy transfer (FRET)-based sensors have been used as an alternative method for imaging calcium signals since they can report changes in protein proximity, which may be altered by binding of calcium (Miyawaki et al., 1997) or of different second messenger molecules. This rationale has been exploited to engineer other FRET-based reporters for other cellular states can be reported by protein proximity, for example: tension. If two labeled protein domains are separated by an elastic linker then FRET can be used as a readout of tension. This rationale has been used to visualize the spatiotemporal organization of forces in live cell imaging experiments (Grashoff et al., 2010).

Protein interactions

Apart from reporting on intra-protein dynamics, FRET is commonly used to understand changes in protein-protein interactions (PPIs). If the donor and acceptor fluorophores are fused to two distinct POIs, their proximity can be reported through donor quenching or acceptor emission. If two POIs are sufficiently close for FRET to occur, they can be inferred to bind (Chudakov et al., 2010).
Dynamic relocalization of proteins can also be used to determine if two distinct POIs interact (see poster – “Protein interaction/Co-rerouting analysis”). The rationale is that if one POI is rerouted to a different cellular location, e.g. mitochondria, and the second POI is co-rerouted, this is evidence for protein-protein interaction (Cheeseman et al., 2013). However, other tests are required to understand if co-rerouting represents a direct protein-protein interaction, if it is via another protein, or even because the two proteins are present on the same cellular structure, which has itself been relocated with the POIs (Larocque et al., 2020). Methods for dynamic relocalization using chemogenetics or optogenetics are covered below. Non-inducible methods to relocalize proteins include tagging a POI with a

targeting motif to ensure its ectopic localization, e.g. targeting Golgins to the mitochondria (Wong and Munro, 2014), Another example of this is the use of nanobodies targeted to a particular location in order to relocalize a POI that is tagged with GFP, for example (Rothbauer et al., 2008; Derivery et al., 2015; Küey et al., 2019).
A further method to visualize PPIs is called Bimolecular Fluorescence Complementation (BiFC) (see poster – "Protein interaction"). It involves tagging two POIs with fragments of a fluorescent protein (FP), which are individually non-fluorescent. If the two proteins come together, it will allow the FP fragments they're tagged with to assemble into a functional FP and the interaction between the POIs can then be observed by microscopy (Hu and Kerppola, 2003).

Inhibition

Inferring a protein's function within a pathway by looking at what happens to this pathway when the POI is inhibited, is a cornerstone of cell biology. To switch to an inhibitory state, two main mechanisms can be deployed; the levels of the POI can be decreased, or the POI can be physically removed from its normal site of activity.

Decreasing protein levels

One mechanism for decreasing proteins levels is by leveraging a system used in plant cells: auxin-induced degradation. Here, the plant auxin hormone indole-3-acetic acid (IAA) is used to promote the interaction between the Skp, cullin, F-box (SCF) complex and an auxin-inducible degron (AID) tag, which is fused to the POI (Verma et al., 2020) (see poster – "Protein degradation"). The SCF complex recruits an E2 ligase, which results in polyubiquitylation of the AID and elimination of the AID-tagged POI by the proteasome. This method acts with a half-life of 20–40 mins (Nishimura et al., 2009), making it far faster than RNAi, but slower than other methods discussed here. A related (non-inducible) method, degradFP uses a nanobody that recognizes GFP to target a GFP-tagged POI to be degraded (Caussinus et al. 2011).

PROteolysis targeting chimeras (PROTACs) that recruit ubiquitin ligases directly to POIs have also been used for targeted destruction and have proven to be a very useful tool for cell biologists (Bondeson et al., 2015; Wang et al., 2021). Here, the PROTAC is designed to include a so-called "warhead" – usually a small molecule that is known to bind selectively to the target – that is used to couple the chimera to the POI (see poster – "Protein degradation"). As this method does not require tagging of the POI or expression of genetically encoded tools, PROTACs can be potentially deployed in native tissues. While this is a strength of the approach, it also has the limitation that the method can only be used where appropriate "warheads" can be developed, and so it is less adaptable compared with other approaches. A further method to inactivate proteins by degradation is Trim-Away (Clift et al., 2017), where an antibody that binds a POI is used to target it for proteasomal degradation. This method works because TRIM21 is a ubiquitin ligase and a receptor that recognizes the introduced antibody. As it relies on an introduced antibody and endogenous factors, this method is particularly useful in cells where expression of genetically encoded tools is difficult. Finally, note that a number of promising approaches to use light for direct protein inactivation (chromophore assisted light inactivation [CALI], KillerRed) have been described, but none are widely utilized yet, suggesting that they may be difficult to use in practice (Ankenbruck et al., 2018).

One of the main chemical heterodimerization tools – the FKBP-rapamycin-FRB system – has also been successfully modified so that it can be used to induce protein degradation. This application uses a modified FK506 binding protein (FKBP) domain fused to the POI and a molecule: called dTAG. dTAG binds both the modified FKBP(F36V) and cereblon, an E3 ubiquitin ligase complex component. Thus, upon dTAG addition into cells the FKBP(F36V)-tagged POI (Nabet et al., 2018; Scheffler et al., 2022) (see poster – "Protein degradation").

Protein relocation

Relocating a protein using induced heterodimerization is a rapid and effective way to inactivate it. In knocksideways, an FKBP-tagged POI is rerouted from its site of action to the mitochondria using an FRB domain attached to the mitochondrial outer membrane protein Tom70p. Upon addition of rapamycin, the POI is trapped at the mitochondria (Robinson et al., 2010) (see poster – "Protein knocksideways"). The rationale is that, since the POI is no longer at its normal site of action, it cannot function and is therefore inactivated. Typically, the endogenous POI is depleted using RNAi so that its function is solely dependent on the overexpressed FKBP-tagged POI (Cheeseman et al., 2013). Alternatively, the FKBP domain can be knocked-in at the endogenous locus using gene editing (Ryan et al., 2021). Rerouting is extremely rapid, on a timescale of seconds to minutes, depending on the intrinsic dynamics of the POI (Robinson et al., 2010). It therefore outperforms degradation methods for cell processes operating on this timescale. In addition to FKBP-rapamycin-FRB, there are several other chemogenetic tools for inducing heterodimerization (see poster – "Main oligomerization tools"), and recently this technology has been further developed to chemically induce heterotrimerization of three POIs by splitting FRB (Wu et al., 2020). Protein inhibition via knocksideways can also be achieved using optogenetics. For example, the microtubule-binding Protein Regulator of Cytokinesis 1 (PRC1), tagged with the bacterial protein SspB, can be co-expressed with iLID, which is targeted to the plasma membrane (PM) by a CAAX motif. Blue light can then be used to inactivate PRC1 by removing it from microtubules onto the plasma membrane through inducing the binding of SspB tag on PRC1 to the PM-anchored iLID. (Jagrić et al., 2021).

There are practical differences between optogenetic and chemogenetic heterodimerization systems that affect their application. First, the illumination can be directed to subcellular regions in order to give tight spatiotemporal control, whereas chemogenetic systems are typically activated throughout the entire cell, and usually across all cells in the cell culture vessel. Second, most chemically-induced heterodimerization systems have long dissociation times (hours), which mean they can be considered irreversible on the timescale of most cell processes. By contrast, optogenetic methods are reversible (typical dissociation time, ~10 s), which allows the cell scientist to observe recovery from inhibition as well as the effect of inhibition itself. It is possible to combine both approaches to allow photocaging of chemical heterodimerization (Ballister et al., 2015) or to add reversibility of chemical heterodimerization using light (Gutnick et al., 2019).

In order to relocate a POI and cause inhibition, the binding that underlies the protein's normal localization must be labile enough to allow its removal (Robinson et al., 2010). Integral membrane proteins, or proteins that bind with very high affinity, cannot be removed in this way. However, if the compartment or structure upon which the POI is located is itself movable, then the entire compartment can be relocalized. This form of inhibition has been demonstrated for the ER (being cleared to the plasma membrane in mitotic cells) and capturing trafficking vesicles on mitochondria (Ferrandiz et al., 2022; Larocque et al., 2021; Hirst et al., 2015).

Activation

The same chemogenetic and optogenetic heterodimerization tools can be deployed to induce activation of a cellular process, termed hotwiring (see poster – “Hotwiring”).
Two classic examples are the induced movement of organelles by coupling them to motors (van Bergeijk et al., 2015), and the recruitment of small GTPases to specific locations to induce their activity (Inoue et al., 2005; Komatsu et al., 2010).
In addition, the formation of clathrin-coated pits can be induced at the plasma membrane to trigger endocytosis on-demand (Wood et al., 2017), achieved by inducible recruitment of a clathrin-binding protein to a plasma membrane anchor. This event can be induced using chemogenetics (FKBP-rapamycin-FRB) or optogenetics (Strickland et al., 2012; Wood et al., 2017). By varying the anchor, it is possible to create clathrin-coated pits on other intracellular membranes, even those that do not normally support pit formation (Küey et al., 2022). Note, that there are many target sequences that can be used to anchor a protein to the desired compartment (for widely used examples, see poster). In many cases, they can be used interchangeably, for example transmembrane proteins CD8a, CD4 or peripheral membrane targeting sequences from Fyn or GAP43 can all be used for triggering endocytosis (Wood et al., 2017).
This rationale of activation using heterodimerization has been exploited in many other creative ways, such as to reactivate the spindle assembly checkpoint (Ballister et al., 2014), to artificially trigger T-cell immune responses (James and Vale, 2012) and to induce selective cell death (Shkarina et al., 2022) (see poster).

Rapamycin activated protease through induced dimerization (RAPID)-release is a novel approach to protein activation, which has been developed to study the nuclear import of histones (Apta Smith *et al.,* 2018). Here, the POI is initially tethered to mitochondria via an OMP25 tag. The POI fusion construct also contains an FRB domain and a cleavage domain that is cut by the tobacco vein mottling virus (TVMV) protease. An FKBP-tagged TVMV protease is co-expressed in this system. Rapamycin addition recruits the protease to the POI through the protease-FKBP-rapamycin-FRB-POI complex formation. There, the protease cuts the cleavage domain and liberates the POI from the mitochondrially-anchored remainder of the fusion construct. This allows the nuclear import of the released POI to be visualized, (half-time, 5 min; Apta Smith *et al.,* 2018) (see poster – “Hotwiring”). RAPID-release is similar to RUSH in allowing the on-demand release of a POI. However, because the initial location of the POI is ectopic rather than within the pathway of interest, we classify this method as ‘activation’ rather than observation.
If it was possible to rapidly produce a POI in cells that do not express it, this could, in principle, be used to activate a pathway it acts in. In practice, inducible expression is too slow (minutes to hours) to be useful for most dynamic cell processes (seconds to minutes). A useful alternative is to continually degrade the POI, before protecting it from degradation in an inducible manner. This can be achieved by tagging a POI with a modified FKBP12 domain, which results in the constitutive degradation of the tagged protein, before adding a compound (Shld1) that binds to FKBP12 and shields the FKBP12-tagged protein from degradation (Banaszynski et al., 2006) (see poster – “Protein stabilisation”).
Most cellular processes are in a dynamic equilibrium; therefore, upregulation of one protein’s activity can be used to inhibit the production of other proteins it interacts with, resulting in the inhibition of downstream pathways in which these interactors function. Two examples, which defy our categorization of inhibition and activation, are the severing of microtubules using opto-katanin (Meiring et al., 2022) and the inhibition of endocytosis by PI(4,5)P2 depletion (Zoncu et al., 2007). In the first case, the microtubule-severing enzyme katanin is

recruited to microtubules using the iLID optogenetic system, causing the localized disassembly of microtubules, which leads to inhibition of transport and other processes (Meiring et al., 2022). In the second, the activation of inositol 5-phosphatase to reduce PI(4,5)P2 levels at the plasma membrane, using chemogenetics, leads to an inhibition of processes which depend on this phosphoinositide, such as endocytosis (Zoncu et al., 2007).

Designing and validating a construct

Almost all of the tools described here require the fusion of protein domains into a single construct for its expression in cells. This modular approach is possible because proteins consist of one or more functional domains, which can be isolated and fused to other domains via short linker sequences, generating new constructs. The design and validation of each new construct is crucial to a successful experiment (see poster – top box “Design and validation of a construct”). In the simplest case, fusion of a fluorescent protein (FP) to a POI, one must consider whether the FP is fused to the N-terminus or C-terminus of the POI. If both N- and C-termini of the POI are known to be important for the protein’s function, or if their orientation means that the tagging would not be functional (e.g. both C- and N- termini of a POI are within the lumen of an organelle, but you want to study a process that takes place in the cytosol), an internal insertion of an FP is possible. Previously, fusions were generated by trial-and-error, but now the structure prediction software AlphaFold can guide the design of fusions (Jumper et al., 2021), by identifying domain boundaries and loops where tags may be inserted. In addition, the length and nature of linker sequences is important. Generally, flexible linkers are favored to allow for protein domains to be independent of one another, but they should only comprise inert amino acid sequences and not contain, for example, sequences for import into cell organelle membranes, which might mistarget the fusion protein inappropriately. Note that long linkers may self-cleave and that glycine-rich linkers may be problematic (Gräwe and Stein, 2020).
Other issues to consider are the properties of the domains to be fused. What is the size of the domain being relative to the size of the POI? For FPs, FPbase is a valuable resource to understand their properties (fluorescent spectra, multimericity, stability etc.) (Lambert, 2019; Cranfill et al., 2016). How the construct will be expressed is a further variable. For some constructs, transient expression with a strong promoter (e.g, CMV) is fine, for others the overexpression may cause problems and a lower-expressing promoter (e.g. PGK) may be necessary. Expression at endogenous levels using gene editing to knock-in additional domains is likely to be the optimal solution (Ryan et al., 2021) but it requires substantially more work. Importantly, each construct must be carefully validated. The simple addition of even a small tag is likely to disrupt the function of a protein to some extent. It is therefore crucial to assess this impact before attempting to use the construct in experiments to test hypotheses about the tagged POIs functions (see poster – top box “Design and validation of a construct”). For instance, whether the localization or dynamics of a POI are affected should be checked in the first instance. Further functional validation can be achieved by testing whether the expression construct can fully complement function in cells depleted of the POI, or whether the expressed protein still interacts with known binding partners.

Conclusions

In this 'Cell science at a glance' and accompanying poster, we highlighted ways that genetically encoded tools can help us to observe cell biological processes and how they can be used to inhibit or activate POIs, allowing us to dissect their roles within these processes. We saw how time is an important factor when dealing with dynamic processes inside the

cell, and that manipulating protein function must be done on a timescale which is at least as fast as the process under study. The past twenty years have seen an explosion in genetically encoded imaging tools, with new variants being constantly reported. Engineering these tools means fusing together proteins in a modular fashion to build constructs for imaging experiments. This process of "remixing" domains is highly creative. The possibilities are vast and novel combinations are driving new insights right now. It is an exciting time to be a cell scientist.

Conflict of interest declaration

The authors declare no competing or financial interests.

Funding

Work in our lab is supported by Cancer Research UK, UK Research and Innovation, and Human Frontier Science Program.

Text boxes

Box 1: The importance of timing

The tools and methods outlined in this article are described as rapidly-acting. This is because each is sufficiently fast so that it is possible to image the cell in the unperturbed state, before applying the respective method, switching state, and observing the resulting behavior. This possibility distinguishes it from slower-acting methods, such as RNAi or knockout, where a correlation of the observation with cellular states is not practical. Moreover, the timescale of many cellular processes is shorter than slower-acting methods (see poster – “Timing”); therefore, rapidly-acting methods are the only feasible way to switch between the perturbed and unperturbed states.

A good example here is in studying cell division. The cell cycle might take 24 h, with M-phase taking up to 1 h. If a slow-acting method such as RNAi, where the POI is degraded over 24-72 h, is used, the function of the protein is difficult to discern as the cell goes through progressive mitoses with reduced levels of the protein. Nevertheless, with proteins that are rapidly turned over, this approach may still be useful. It is also possible that loss of the protein through RNAi has additional unintended consequences. A rapid inactivation method, where the protein is switched off immediately before (or during) mitosis, thus allows a more unambiguous interpretation of phenotype (Royle, 2013). Note that even the most rapid methods described here (optogenetics) can also work on longer timescales, so they should not be discounted as options when studying slower cell biological processes.

Box 2 inducible heterodimerization

Heterodimerization of two genetically encoded proteins can be induced using chemical or optical means. In the widest used chemogenetic method, the FKBP domain of FKBP12 is fused to one protein and a FKBP-rapamycin binding (FRB) domain of mTOR is fused to another. Rapamycin can then be applied, which binds to FKBP and together the FKBP-rapamycin complex binds tightly to FRB (K_d, 10 nM), causing heterodimerization of the two proteins tagged with FRB and FKBP domains (Spencer et al., 1993). Rapamycin derivatives (rapalogs), such as AP21967, do not interact with endogenous domains that bind rapamycin, and can be used with modified FRB domains, for example FRB(T2098L), to reduce the potentially confounding effects of rapamycin on cell physiology. One example of a reversible chemically-induced heterodimerization system involves the coupling of a modified FKBP domain (F36V) to a non-FRB domain (eDHFR) via a synthetic dimerising agent SLF’-TMP (Voß et al., 2015). An alternative widely used chemical heterodimerization method uses GA3-AM, a gibberellin (plant hormone) derivative which binds receptor gibberellin insensitive dwarf1 (GID1). GA3-AM binding causes a conformational change in GID that permits it to bind to gibberellin insensitive (GAI), which would induce the dimerization between proteins tagged with GID and GAI domains, There are further variations on this principle (see poster – “Main oligomerization tools”).

A myriad of optogenetic heterodimerization systems have also been developed (Benedetti, 2021), including CRY2/CIB1 (Kennedy et al., 2010), tunable light-interacting proteins (TULIPs, Strickland et al., 2012), improved light-inducible dimer (iLID, Guntas et al., 2015), eMags (Benedetti et al., 2020) and others (see poster – “Main oligomerization tools”) documented at optobase (Kolar et al., 2018). Generally, these methods include a light-sensitive domain, which binds another domain upon illumination with light of a certain wavelength, so they can be used for heterodimerization in an analogous manner to chemogenetic methods. Both chemical and optical modalities give the cell scientist control over heterodimerization in their experiment. They can even be combined in the case of

DHFR/zapalog to chemically induce heterodimerization and then, by use of light, dissociate the induced dimer.

References

Apta-Smith, M. J., **Hernandez-Fernaud, J. R.**, and **Bowman, A. J.** (2018). Evidence for the nuclear import of histones H3.1 and H4 as monomers. *EMBO J*, 37(19):e98714. doi: 10.15252/embj.201798714.

Ankenbruck, N., Courtney, T., Naro, Y., and **Deiters, A.** (2018). Optochemical Control of Biological Processes in Cells and Animals. *Angew Chem Int Ed Engl*, 57: 2768–2798. doi: 10.1002/anie.201700171.

Ballister, E. R., **Riegman, M.**, and **Lampson, M. A.** (2014). Recruitment of Mad1 to metaphase kinetochores is sufficient to reactivate the mitotic checkpoint. *J Cell Biol*, 204(6):901–908. doi: 10.1083/jcb.201311113.

Ballister, E. R., **Ayloo, S.**, **Chenoweth, D. M.**, **Lampson, M. A.**, and **Holzbaur, E. L. F.** (2015). Optogenetic control of organelle transport using a photocaged chemical inducer of dimerization. *Curr Biol*, 25(10): R407–R408. doi: 10.1016/j.cub.2015.03.056.

Banaszynski, L. A., **Chen, L.-C.**, **Maynard-Smith, L. A.**, **Ooi, A. G. L.**, and **Wandless, T. J.** (2006). A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell*, 126(5):995–1004. doi: 10.1016/j.cell.2006.07.025.

Benedetti, L., Marvin, J.S., Falahati, H., Guillén-Samander, A., Looger, L.L., and **De Camilli, P.** (2020). Optimized Vivid-derived Magnets photodimerizers for subcellular optogenetics in mammalian cells. *Elife*, 9: e63230. doi: 10.7554/eLife.63230.

Benedetti, L. (2021). Optogenetic Tools for Manipulating Protein Subcellular Localization and Intracellular Signaling at Organelle Contact Sites. *Curr Protoc*, 1(3):e71. doi: 10.1002/cpz1.71.

Boncompain, G., **Divoux, S.**, **Gareil, N.**, **de Forges, H.**, **Lescure, A.**, **Latreche, L.**, **Mercanti, V.**, **Jollivet, F.**, **Raposo, G.**, and **Perez, F.** (2012). Synchronization of secretory protein traffic in populations of cells. *Nat Methods*, 9(5):493–498. doi: 10.1038/nmeth.1928.

Bondeson, D. P., **Mares, A.**, **Smith, I. E. D.**, **Ko, E.**, **Campos, S.**, **Miah, A. H.**, **Mulholland, K. E.**, **Routly, N.**, **Buckley, D. L.**, **Gustafson, J. L.**, **Zinn, N.**, **Grandi, P.**, **Shimamura, S.**, **Bergamini, G.**, **Faelth-Savitski, M.**, **Bantscheff, M.**, **Cox, C.**, **Gordon, D. A.**, **Willard, R. R.**, **Flanagan, J. J.**, **Casillas, L. N.**, **Votta, B. J.**, **den Besten, W.**, **Famm, K.**, **Kruidenier, L.**, **Carter, P. S.**, **Harling, J. D.**, **Churcher, I.**, and **Crews, C. M.** (2015). Catalytic in vivo protein knockdown by small-molecule PROTACs. *Nat Chem Biol*, 11(8):611–617. doi: 10.1038/nchembio.1858.

Caussinus, E., Kanca, O., and **Affolter, M.** (2011). Fluorescent fusion protein knockout mediated by anti-GFP nanobody. *Nat Struct Mol Biol*, 19: 117–121. doi: 10.1038/nsmb.2180.

Cheeseman, L. P., **Harry, E. F.**, **McAinsh, A. D.**, **Prior, I. A.**, and **Royle, S. J.** (2013). Specific removal of TACC3-ch-TOG-clathrin at metaphase deregulates kinetochore fiber tension. *J Cell Sci*, 126(Pt 9): 2102–13. doi: 10.1242/jcs.124834.

Chen, T.-W., **Wardill, T. J.**, **Sun, Y.**, **Pulver, S. R.**, **Renninger, S. L.**, **Baohan, A.**, **Schreiter, E. R.**, **Kerr, R. A.**, **Orger, M. B.**, **Jayaraman, V.**, **Looger, L. L.**, **Svoboda, K.**, and **Kim, D. S.** (2013). Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature*, 499(7458):295–300. doi: 10.1038/nature12354.

Chudakov, D. M., **Matz, M. V.**, **Lukyanov, S.**, and **Lukyanov, K. A.** (2010). Fluorescent proteins and their applications in imaging living cells and tissues. *Physiol Rev*, 90(3):1103–1163. doi: 10.1152/physrev.00038. 2009.

Clarke, N. I. and **Royle, S. J.** (2018). FerriTag is a new geneticallyencoded inducible tag for correlative light-electron microscopy. *Nat Commun*, 9(1):2604. doi: 10.1038/s41467-018-04993-0.

Clift, D., **McEwan, W. A.**, **Labzin, L. I.**, **Konieczny, V.**, **Mogessie, B.**, **James, L. C.**, and **Schuh, M.** (2017). A Method for the Acute and Rapid Degradation of Endogenous Proteins. *Cell*, 171(7):1692–1706.e18. doi: 10.1016/j.cell.2017.10.033.

Cranfill, P.J., Sell, B.R., Baird, M.A., Allen, J.R., Lavagnino, Z., de Gruiter, H.M., Kremers, G.-J., Davidson, M.W., Ustione, A. and **Piston, D.W.** (2016) Quantitative assessment of fluorescent proteins. *Nat Methods* 13: 557–562. doi: 10.1038/nmeth.3891

Derivery, E., **Seum, C.**, **Daeden, A.**, **Loub´ery, S.**, **Holtzer, L.**, **Jülicher, F.**, and **Gonzalez-Gaitan, M.** (2015). Polarized endosome dynamics by spindle asymmetry during asymmetric cell division. *Nature*, 528(7581):280–285. doi: 10.1038/nature16443.

Ferrandiz, N., **Downie, L.**, **Starling, G. P.**, and **Royle, S. J.** (2022). Endomembranes promote chromosome missegregation by ensheathing misaligned chromosomes. *J Cell Biol*, 221(6):e202203021. doi: 10.1083/jcb.202203021.

Gräwe, A., and **Stein, V.** (2021). Linker Engineering in the Context of Synthetic Protein Switches and Sensors. *Trends Biotechnol*, 39:731–744. doi: 10.1016/j.tibtech.2020.11.007.

Grashoff, C., **Hoffman, B. D.**, **Brenner, M. D.**, **Zhou, R.**, **Parsons, M.**, **Yang, M. T.**, **McLean, M. A.**, **Sligar, S. G.**, **Chen, C. S.**, **Ha, T.**, and **Schwartz, M. A.** (2010). Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature*, 466(7303): 263–266. doi: 10.1038/nature09198.

Guntas, G., Hallett, R.A., Zimmerman, S.P., Williams, T., Yumerefendi, H., Bear, J.E., and **Kuhlman, B.** (2015). Engineering an improved light-induced dimer (iLID) for controlling the localization and activity of signaling proteins. *Proc Natl Acad Sci U S A*, 112: 112–117. doi: 10.1073/pnas.1417910112.

Gutnick, A., **Banghart, M. R.**, **West, E. R.**, and **Schwarz, T. L.** (2019). The light-sensitive dimerizer zapalog reveals distinct modes of immobilization for axonal mitochondria. *Nat Cell Biol*, 21(6):768–777. doi: 10.1038/s41556-019-0317-2.

Hirst, J., **Edgar, J. R.**, **Borner, G. H. H.**, **Li, S.**, **Sahlender, D. A.**, **Antrobus, R.**, and **Robinson, M. S.** (2015). Contributions of epsinR and gadkin to clathrin-mediated intracellular trafficking. *Mol Biol Cell*, 26(17):3085–3103. doi: 10.1091/mbc.E15-04-0245.

Hu, C.-D. and **Kerppola, T. K.** (2003). Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat Biotechnol*, 21(5):539–545. doi: 10.1038/ nbt816.

Huet-Calderwood, C., **Rivera-Molina, F.**, **Iwamoto, D. V.**, **Kromann, E. B.**, **Toomre, D.**, and **Calderwood, D. A.** (2017). Novel ecto-tagged integrins reveal their trafficking in live cells. *Nat Commun*, 8 (1):570. doi: 10.1038/s41467-017-00646-w.

Inoue, T., Heo, W.D., Grimley, J.S., Wandless, T.J., and **Meyer, T.** (2005). An inducible translocation strategy to rapidly activate and inhibit small GTPase signaling pathways. *Nat Methods*, 2: 415–418. doi: 10.1038/nmeth763.

Jagrić, M., **Risteski, P.**, **Martinčić, J.**, **Milas, A.**, and **Tolić, I. M.** (2021). Optogenetic control of PRC1 reveals its role in chromosome alignment on the spindle by overlap length-dependent forces. *Elife*, 10:e61170. doi: 10.7554/eLife.61170.

James, J. R. and **Vale, R. D.** (2012). Biophysical mechanism of T-cell receptor triggering in a reconstituted system. *Nature*, 487(7405):64–69. doi: 10.1038/nature11220.

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., et al. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature*, 596:583–589. doi: 10.1038/s41586-021-03819-2.

Kennedy, M.J., Hughes, R.M., Peteya, L.A., Schwartz, J.W., Ehlers, M.D., and **Tucker, C.L.** (2010). Rapid blue-light-mediated induction of protein interactions in living cells. *Nat Methods*, 7: 973–975. doi: 10.1038/nmeth.1524.

Kolar, K., Knobloch, C., Stork, H., Žnidarič, M., and **Weber, W.** (2018). OptoBase: A Web Platform for Molecular Optogenetics. *ACS Synth Biol*, 7: 1825–1828. doi: 10.1021/acssynbio.8b00120.

Komatsu, T., **Kukelyansky, I.**, **McCaffery, J. M.**, **Ueno, T.**, **Varela, L. C.**, and **Inoue, T.** (2010). Organelle-specific, rapid induction of molecular activities and membrane tethering. *Nat Methods*, 7(3):206–208. doi: 10.1038/nmeth.1428.

Küey, C., **Larocque, G.**, **Clarke, N. I.**, and **Royle, S. J.** (2019). Unintended perturbation of protein function using GFP nanobodies in human cells. *J Cell Sci*, 132(21):jcs234955. doi: 10.1242/jcs.234955.

Küey, C., **Sittewelle, M.**, **Larocque, G.**, **Hern´andez-Gonz´alez, M.**, and **Royle, S. J.** (2022). Recruitment of clathrin to intracellular membranes is sufficient for vesicle formation. *Elife*, 11:e78929. doi: 10.7554/eLife.78929.

Lambert, T. J. (2019). FPbase: a community-editable fluorescent protein database. *Nat Methods*, 16(4):277–278. doi: 10.1038/s41592-019-0352-8.

Larocque, G., **La-Borde, P. J.**, **Clarke, N. I.**, **Carter, N. J.**, and **Royle, S. J.** (2020). Tumor protein D54 defines a new class of intracellular transport vesicles. *J Cell Biol*, 219(1):e201812044. doi: 10.1083/jcb.201812044.

Larocque, G., **Moore, D. J.**, **Sittewelle, M.**, **Kuey, C.**, **Hetmanski, J. H. R.**, **La-Borde, P. J.**, **Wilson, B. J.**, **Clarke, N. I.**, **Caswell, P. T.**, and **Royle, S. J.** (2021). Intracellular nanovesicles mediate *α*5*β*1 integrin trafficking during cell migration. *J Cell Biol*, 220(10):e202009028. doi: 10.1083/jcb.202009028.

McCaughey, J., Stevenson, N.L., Cross, S., and **Stephens, D.J.** (2019). ER-to-Golgi trafficking of procollagen in the absence of large carriers. *J Cell Biol*, 218: 929–948. doi: 10.1083/jcb.201806035.

Meiring, J. C. M., **Grigoriev, I.**, **Nijenhuis, W.**, **Kapitein, L. C.**, and **Akhmanova, A.** (2022). Opto-katanin, an optogenetic tool for localized, microtubule disassembly. *Curr Biol*, pages S0960–9822(22)01454–3. doi: 10.1016/j.cub.2022.09.010.

Merrifield, C. J., **Perrais, D.**, and **Zenisek, D.** (2005). Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. *Cell*, 121(4):593–606. doi: 10.1016/j.cell.2005.03.015.

Miesenböck, G., **De Angelis, D. A.**, and **Rothman, J. E.** (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*, 394(6689):192–195. doi: 10.1038/28190.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., and **Tsien, R.Y.** (1997). Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. *Nature*, 388: 882–887. Doi: 10.1038/42264.

Nabet, B., **Roberts, J. M.**, **Buckley, D. L.**, **Paulk, J.**, **Dastjerdi, S.**, **Yang, A.**, **Leggett, A. L.**, **Erb, M. A.**, **Lawlor, M. A.**, **Souza, A.**, **Scott, T. G.**, **Vittori, S.**, **Perry, J. A.**, **Qi, J.**, **Winter, G. E.**, **Wong, K.-K.**, **Gray, N. S.**, and **Bradner, J. E.** (2018). The dTAG system for immediate and target-specific protein degradation. *Nat Chem Biol*, 14(5):431–441. doi: 10.1038/s41589-018-0021-8.

Nishimura, K., **Fukagawa, T.**, **Takisawa, H.**, **Kakimoto, T.**, and **Kanemaki, M.** (2009). An auxin-based degron system for the rapid depletion of proteins in nonplant cells. *Nat Methods*, 6(12):917–922. doi: 10.1038/nmeth.1401.

Phuyal, S., Djaerff, E., Le Roux, A.-L., Baker, M.J., Fankhauser, D., Mahdizadeh, S.J., Reiterer, V., Parizadeh, A., Felder, E., Kahlhofer, J.C., et al. (2022). Mechanical strain

stimulates COPII-dependent secretory trafficking via Rac1. *EMBO J*, 41: e110596. doi: 10.15252/embj.2022110596.

Robinson, M. S., **Sahlender, D. A.**, and **Foster, S. D.** (2010). Rapid inactivation of proteins by rapamycin-induced rerouting to mitochondria. *Dev Cell*, 18(2):324–331. doi: 10.1016/j.devcel.2009.12.015.

Rothbauer, U., Zolghadr, K., Muyldermans, S., Schepers, A., Cardoso, M.C., and **Leonhardt, H.** (2008). A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. *Mol Cell Proteomics*, 7: 282–289. doi: 10.1074/mcp.M700342-MCP200.

Royle, S. J. (2013). Protein adaptation: mitotic functions for membrane trafficking proteins. *Nat Rev Mol Cell Biol*, 14(9):592–9. doi: 10.1038/ nrm3641.

Ryan, E. L., **Shelford, J.**, **Massam-Wu, T.**, **Bayliss, R.**, and **Royle, S. J.** (2021). Defining endogenous TACC3-chTOG-clathrin-GTSE1 interactions at the mitotic spindle using induced relocalization. *J Cell Sci*, 134(3). doi: 10.1242/jcs.255794.

Sakaue-Sawano, A., **Kurokawa, H.**, **Morimura, T.**, **Hanyu, A.**, **Hama, H.**, **Osawa, H.**, **Kashiwagi, S.**, **Fukami, K.**, **Miyata, T.**, **Miyoshi, H.**, **Imamura, T.**, **Ogawa, M.**, **Masai, H.**, and **Miyawaki, A.** (2008). Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell*, 132(3):487–498. doi: 10.1016/j.cell.2007.12.033.

Scheffler, K., **Giannini, F.**, **Lemonnier, T.**, and **Mogessie, B.** (2022). The prophase oocyte nucleus is a homeostatic G-actin buffer. *J Cell Sci*, 135(6):jcs259807. doi: 10.1242/jcs.259807.

Shkarina, K., **Hasel de Carvalho, E.**, **Santos, J. C.**, **Ramos, S.**, **Leptin, M.**, and **Broz, P.** (2022). Optogenetic activators of apoptosis, necroptosis, and pyroptosis. *J Cell Biol*, 221(6):e202109038. doi: 10.1083/ jcb.202109038.

Spencer, D.M., Wandless, T.J., Schreiber, S.L., and **Crabtree, G.R.** (1993). Controlling signal transduction with synthetic ligands. *Science*, 262: 1019–1024. doi: 10.1126/science.7694365

Strickland, D., **Lin, Y.**, **Wagner, E.**, **Hope, C. M.**, **Zayner, J.**, **Antoniou, C.**, **Sosnick, T. R.**, **Weiss, E. L.**, and **Glotzer, M.** (2012). TULIPs: tunable, light-controlled interacting protein tags for cell biology. *Nat Methods*, 9(4):379–384. doi: 10.1038/nmeth.1904.

van Bergeijk, P., **Adrian, M.**, **Hoogenraad, C. C.**, and **Kapitein, L. C.** (2015). Optogenetic control of organelle transport and positioning. *Nature*, 518(7537):111–114. doi: 10.1038/nature14128.

Verma, R., Mohl, D., and **Deshaies, R.J.** (2020). Harnessing the Power of Proteolysis for Targeted Protein Inactivation. *Mol Cell*, 77: 446–460. doi: 10.1016/j.molcel.2020.01.010.

Voß, S., **Klewer, L.**, and **Wu, Y.-W.** (2015). Chemically induced dimerization: reversible and spatiotemporal control of protein function in cells. *Curr Opin Chem Biol*, 28:194–201. doi: 10.1016/j.cbpa.2015.09.003.

Wang, R., **Ascanelli, C.**, **Abdelbaki, A.**, **Fung, A.**, **Rasmusson, T.**, **Michaelides, I.**, **Roberts, K.**, and **Lindon, C.** (2021). Selective targeting of non-centrosomal AURKA functions through use of a targeted protein degradation tool. *Commun Biol*, 4(1):640. doi: 10.1038/s42003-021-02158-2.

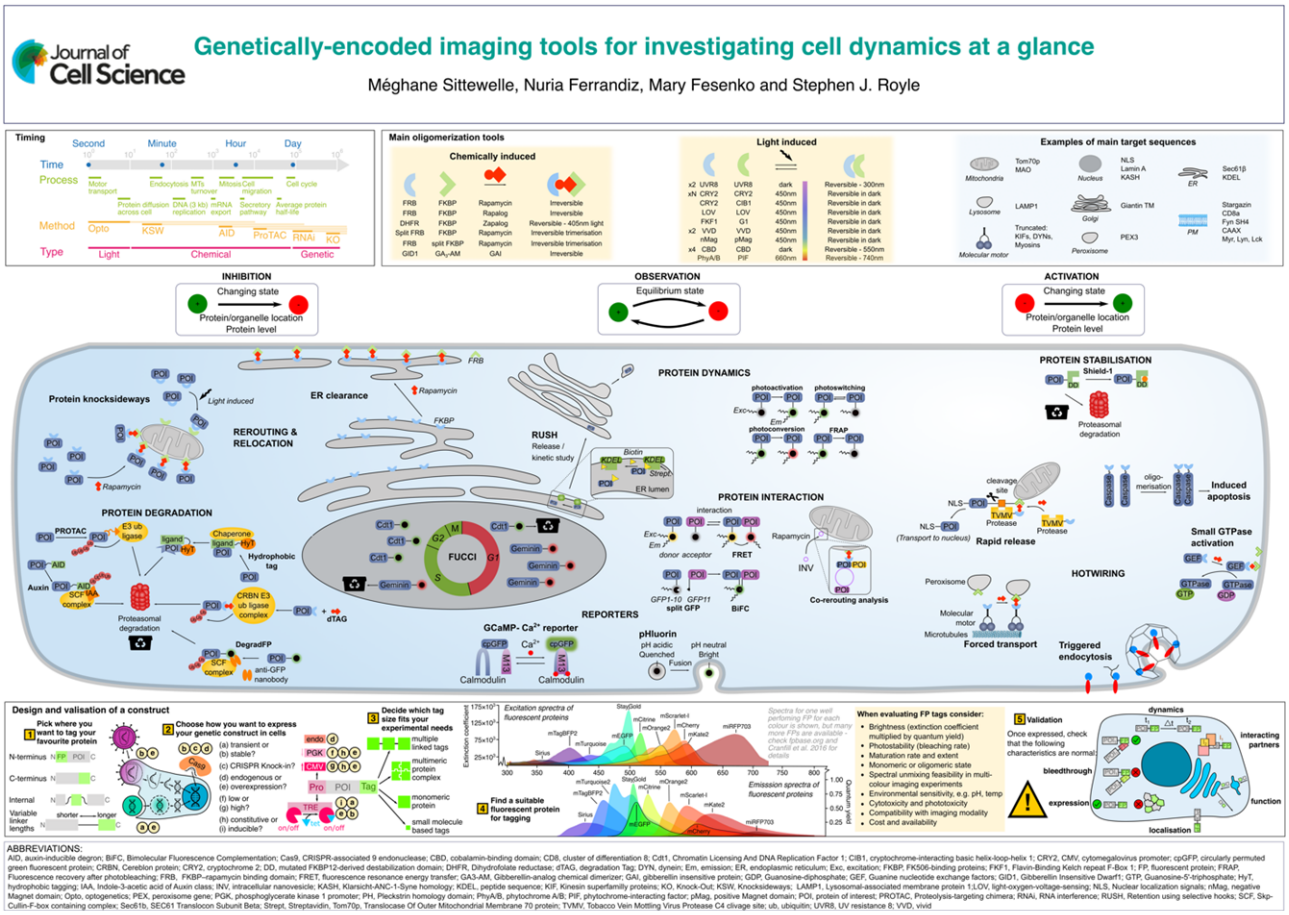
Wong, M. and **Munro, S.** (2014). Membrane trafficking. The specificity of vesicle traffic to the Golgi is encoded in the golgin coiled-coil proteins. *Science*, 346(6209):1256898. doi: 10.1126/science.1256898.

Wood, L. A., **Larocque, G.**, **Clarke, N. I.**, **Sarkar, S.**, and **Royle, S. J.** (2017). New tools for ”hot-wiring” clathrin-mediated endocytosis with temporal and spatial precision. *J. Cell Biol.*, 216(12):4351–4365. doi: 10.1083/jcb.201702188.

Wu, H. D., **Kikuchi, M.**, **Dagliyan, O.**, **Aragaki, A. K.**, **Nakamura, H.**, **Dokholyan, N. V.**, **Umehara, T.**, and **Inoue, T.** (2020). Rational design and implementation of a chemically

inducible heterotrimerization system. *Nat Methods*, 17(9):928–936. doi: 10.1038/s41592-020-0913-x.

Zoncu, R., **Perera, R. M.**, **Sebastian, R.**, **Nakatsu, F.**, **Chen, H.**, **Balla, T.**, **Ayala, G.**, **Toomre, D.**, and **De Camilli, P. V.** (2007). Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. *Proc Natl Acad Sci U S A*, 104(10): 3793–3798. doi: 10.1073/pnas.0611733104.



ABBREVIATIONS:
AID, auxin-inducible degron; BiFC, Bimolecular Fluorescence Complementation; Cas9, CRISPR-associated 9 endonuclease; CBD, cobalamin-binding domain; CD8, cluster of differentiation 8; Cdt1, Chromatin Licensing And DNA Replication Factor 1; CIB1, cryptochrome-interacting basic helix-loop-helix 1; CRY2, CMV, cytomegalovirus promoter; cpGFP, circularly permuted green fluorescent protein; CRBN, Cereblon protein; CRY2, cryptochrome 2; DD, mutated FKBP12-derived destabilization domain; DHFR, Dihydrofolate reductase; dTAG, degradation Tag; DYN, dynein; Em, emission; ER, endoplasmic reticulum; Exc, excitation; FKBP, FK506-binding proteins; FKF1, Flavin-Binding Kelch repeat F-Box 1; FP, fluorescent protein; FRAP, Fluorescence recovery after photobleaching; FRB, FKBP–rapamycin binding domain; FRET, fluorescence resonance energy transfer; GA3-AM, Gibberellin-analog chemical dimerizer; GAI, gibberellin insensitive protein; GDP, Guanosine-diphosphate; GEF, Guanine nucleotide exchange factors; GID1, Gibberellin Insensitive Dwarf1; GTP, Guanosine-5′-triphosphate; HyT, hydrophobic tagging; IAA, Indole-3-acetic acid of Auxin class; INV, intracellular nanovesicle; KASH, Klarsicht-ANC-1-Syne homology; KDEL, peptide sequence; KIF, Kinesin superfamily proteins; KO, Knock-Out; KSW, Knocksideways; LAMP1, Lysosomal-associated membrane protein 1;LOV, light-oxygen-voltage-sensing; NLS, Nuclear localization signals; nMag, negative Magnet domain; Opto, optogenetics; PEX, peroxisome gene; PGK, phosphoglycerate kinase 1 promoter; PH, Pleckstrin homology domain; PhyA/B, phytochrome A/B; PIF, phytochrome-interacting factor; pMag, positive Magnet domain; POI, protein of interest; PROTAC, Proteolysis-targeting chimera; RNAi, RNA interference; RUSH, Retention using selective hooks; SCF, Skp-Cullin-F-box containing complex; Sec61b, SEC61 Translocon Subunit Beta; Strept, Streptavidin, Tom70p, Translocase Of Outer Mitochondrial Membrane 70 protein; TVMV, Tobacco Vein Mottling Virus Protease C4 clivage site; ub, ubiquitin; UVR8, UV resistance 8; VVD, vivid